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Antihyperalgesic effects of a novel muscarinic agonist (LASSBio-873) in spinal nerve ligation in rats

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SUMMARY

1. New chemicals or adjuvants with analgesic effects on chronic pain are needed and clinically relevant due to the limited number of effective compounds that possess these characteristics. LASSBio-873, a pyrazolo[3,4-*b*]pyrrolo[3,4-*d*]pyridine derivative, activates muscarinic cholinergic receptors and has potent analgesic effects on acute and inflammatory pain.

2. The present study evaluated the therapeutic and prophylactic effects of oral administration of LASSBio-873 in a spinal nerve ligation (SNL) model of chronic peripheral nerve injury.

3. LASSBio-873 (100 mg/kg) inhibited the development of thermal hyperalgesia and mechanical allodynia when administered once daily for 7 consecutive days after SNL surgery and reversed these symptoms. LASSBio-873 treatment did not alter rat behaviour in open field testing measured during the first 24 h after administration and again after 7 continuous days administration. The analgesic effect of LASSBio-873 was inhibited by intrathecal methoctramine, an M_2 receptor antagonist, implicating the muscarininc M_2 receptor signalling pathway in the drug's action.

4. These results reinforce the potential of LASSBio-873 as a possible prototype for the development of more effective alternatives for the treatment of neuropathic pain.

Key words: Pyrazolo[3,4-b]pyrrolo[3,4-d]pyridine derivatives, chronic pain, hyperalgesia, muscarinic, spinal nerve ligation.

INTRODUCTION

Pain in humans is a significant health and economic issue. The combined cost of pain treatment in developed countries averages US$1 trillion per year.[1] In the US alone, medical and lost productivity costs associated with chronic pain total as much as US $635 billion annually.[2]

The International Association for the Study of Pain (IASP) defines neuropathic pain as pain caused by a lesion or disease of the somatosensory nervous system.[3,4] Different kinds of insults to the nervous system can stimulate the development of neuropathic pain. The mechanisms involved in the development and maintenance of pain states involving the spinal cord are not fully understood.[5]

A high prevalence of chronic neuropathic pain that severely impairs the quality of life of affected patients has been observed in clinical practice.[6] The spectrum of neuropathic pain covers a variety of disease states, including diabetes mellitus, herpes zoster, nerve compression or radiculopathy, alcoholism, chemotherapy, drug abuse and trigeminal neuralgia, which are associated with allodynia and hyperalgesia.[7–9]

The drug classes providing clinically effective treatment of neuropathic pain include tricyclic antidepressants, serotonin and noradrenaline reuptake inhibitors, the anticonvulsants gabapentin and pregabalin and opioids.[8,10,11] Opioids are generally used to treat chronic pain only after at least two other approaches have failed. Current clinical treatments for neuropathic pain include amitriptyline, a tricyclic antidepressant with mixed pharmacology that can impair cognitive performance, and gabapentin, a compound that interacts selectively with $\alpha 2\delta 1$ calcium channel subunits.[12]

The use of multiple drug therapies is common in clinical practice. Despite a considerable increase in the number of randomized placebo-controlled trials investigating potential therapies for neuropathic pain, the medical treatments that are available are far from satisfactory, with less than 50% of patients significantly benefiting from any pharmacological agent.[8] Adverse side-effects and insufficient efficacy have been the driving forces behind the development of new analgesics.

Novel substances, collectively referred to as pyrazolo[3,4-*b*]pyrrolo[3,4-*d*]pyridine derivatives, have been designed and synthesised from zolpidem as a lead compound. These derivatives were originally designed to be selective GABA receptor modulators based on their structural analogy with zolpidem, which produces rapid-onset, short-duration hypnosis as a consequence of binding to benzodiazepine receptors.[9] Zolpidem induces analgesia at high doses (80 mg/kg),[8] but has the secondary effect

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of decreasing locomotor activity in mice.[13–17] Although the mechanisms responsible for zolpidem-induced analgesia are not yet known, opioid pathway involvement has been suggested.[8]

Of the zolpidem derivatives tested, LASSBio-873 has been shown to be the most effective as an analgesic.[18] The antinociceptive activity of LASSBio-873 has been shown to be mediated via activation of the muscarinic system and it reduces carrageenan-induced thermal hyperalgesia as well as formalin-induced acute inflammatory pain.[19] Unlike the parent compound zolpidem, the pharmacological activities of LASSBio-873 were not affected by flumazenil (a benzodiazepine receptor antagonist) or yohimbine (an α_2-adrenoceptor antagonist), indicating that muscarinic acetylcholine receptor pathways are selectively important for the drug's analgesic activity.[19]

Based on the results of the studies discussed above, it was considered of importance to investigate the efficacy of LASSBio-873 in the treatment and prevention of neuropathic pain. Therefore, the main objective of the present study was to investigate the prophylactic and therapeutic effects of LASSBio-873 in neuropathic pain, using the experimental model of spinal nerve ligation (SNL) in rats.[20] In this model, as in most animal models of neuropathic pain, nerve injury leads to varying degrees of hypersensitivity to tactile stimuli, chemicals and heat, with signs of pain that, much like clinically observed neuropathic pain, do not resolve with traditional analgesics.[21] In addition, we tested the effect of LASSBio-873 on animals' motor behaviour and examined whether the analgesic effects of LASSBio-873 are mediated through muscarinic M_2 receptor signalling.

METHODS

Animals

The present study was approved by the Animal Care and Use Committee at Universidade Federal do Rio de Janeiro (UFRJ; Rio de Janeiro, Brazil). Male Wistar rats (*Rattus norvegicus*) provided by the animal facility of the UFRJ, weighing 160–200 g, were maintained in a temperature (25°C)- and humidity (50–60%)-controlled room on a 12 h light–dark cycle. Water and food were available *ad libitum*. Animals were acclimated to the laboratory environment for at least 40 min before the start of experiments. Each animal was used for one experiment only.

Drugs

LASSBio-873 was synthesised and provided by the Laboratório de Avaliação e Síntese de Substâncias Bioativas (UFRJ). Methoctramine was purchased from Sigma (St Louis, MO, USA). Morphine sulphate, dimethylsulphoxide (DMSO) and nanoemulsion were kindly donated by Cristalia Produtos Químicos e Farmacêuticos (Itapira, Brazil) and were used in the vehicle solution.

Surgery

Spinal nerve ligation was used to induce peripheral neuropathy.[20] Briefly, after rats had been anaesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.), their skin was sterilized with 0.5% chlorhexidine and the lateral laminae of the lower lumbar and upper sacral vertebrae were exposed by a dorsal incision. The right L6 transverse process was removed and the right L5 spinal nerve was isolated and ligated tightly using 6–0 silk sutures. For sham-operated rats, the same process was performed except that the spinal nerve was not ligated.

Somatosensory and motor testing

Heat withdrawal latency

Heat withdrawal latency was assessed by applying a focused radiant heat source (produced by a light beam) to the hind paw through a glass plate.[22] The light beam was deactivated when the animal lifted its hind paw, allowing measurement of the length of time the paw remained in contact with the heat source. Withdrawal latency was determined using a plantar analgesia meter (model 33; ITC, Woodland Hills, CA, USA). The latency to evoke withdrawal was determined with a cut-off value of 30 s. Control latency was determined using the average response of three measurements separated by 5 min intervals prior to the surgical procedure. Both hind paws were tested. Nociceptive threshold, as determined by heat withdrawal latency, was measured prior to and on after surgery. Animals were excluded if a motor deficit was observed.

Mechanical allodynia

Withdrawal threshold to pressure applied to the hind paw (in g) was measured using a digital analgesimeter (model EFF301; Insight, Ribeirão Preto, Brazil).[23] The pressure meter consisted of a hand-held force transducer fitted with a 0.7 mm^2 polypropylene tip. Upon paw withdrawal, the pressure was released immediately and the nociceptive threshold was read on a scale. Stimulation of the paw was repeated five times and the readings were accepted if the difference between the highest and lowest measure was < 10 g. An upper limit of 150 g was used to avoid potential tissue injury in the absence of a response. Mechanical allodynia and thermal hyperalgesia were evaluated on the same day.

Locomotor activity test

Sedative activity was investigated by recording spontaneous locomotor activity in a 45×45 cm open field chamber (LE 8811; Letica, Barcelona, Spain) in which 16 infrared photocells were positioned every 2.5 cm. Total locomotor activity was defined as the number of beam interruptions registered in the attached computer during the 40 min period following oral administration of either vehicle or LASSBio-873 (100 mg/kg) in a group of 10 rats. Data are expressed as the number of movements per minute. This evaluation was conducted immediately and 24 h after a single administration or after 7 consecutive days of substance administration. This procedure was applied to control for sedative activity in the evaluation of postoperative hyperalgesia and allodynia.

Dose–response curve

Animals subjected to SNL surgery, as described above, were observed for 7 days and animals that then demonstrated the

development of neuropathy were divided randomly into groups treated by gavage with 0, 8, 20, 40, 75 or 100 mg/kg, p.o., LASSBio-873 for 7 consecutive days. Paw withdrawal threshold response in the heat hyperalgesia test was evaluated at the end of treatment and latency was plotted using linear regression.

Dosing protocols

Prophylactic protocol

Thirty-two Wistar rats were randomly divided into sham and SNL groups. A single dose of either vehicle or LASSBio-873 was administered orally 4 h after surgery by gavage and then again on each of the 7 consecutive days after surgery. The four dosing experimental groups were: SNL/DMSO-p.o., SHAM/DMSO-p.o., SNL/LASSBio-873-p.o and SHAM/LASSBio-873-p.o. The neuropathic (SNL/DMSO) and non-neuropathic groups (SHAM/DMSO) were treated with 1.5 μL/g bodyweight, p.o., DMSO for 7 days, whereas the SNL/LASSBio-873-p.o and SHAM/LASSBio-873-p.o. groups were treated with 100 mg/kg, p.o., LASSBio-873 for 7 days.

Therapeutic protocol

Seven days after surgery, SNL animals with a paw withdrawal response significantly lower than the response of control animals were considered neuropathic. Treatment was then initiated and lasted 7 days (from Day 7 to Day 14 after surgery), with sham and SNL groups subdivided as in the protocol described above.

Intrathecal injection

Sevoflurane (3%)-anaesthetized rats were injected intrathecally with 10 μg methoctramine, morphine (reference analgesic), LASSBio-873 or 40 μL vehicle. Rats (n = 6 per group) were positioned for intrathecal injection at the L4–5 intervertebral space using a 30 gauge needle attached to a 300 μL syringe. The tail flick reaction was used to confirm proper positioning of the needle in the intrathecal space. A 40 μL aliquot of drug solution was injected into each animal. Control animals received intrathecal vehicle (nanoemulsion) only. Animals were allowed to recover from anaesthesia for 10 min before starting the nociceptive test. In this protocol, DMSO was not used as a vehicle due to its potential activity.

Experimental protocols

Prophylactic protocol

Thermal hyperalgesia and mechanical allodynia were measured on post-surgical Days 3 and 7. Baseline (BL) measures were evaluated immediately before surgery.

Therapeutic protocol

Thermal hyperalgesia and mechanical allodynia were measured on post-surgical Days 10 and 14, corresponding to post-treatment Days 3 and 7. Baseline measures were evaluated immediately before the surgery.

Intrathecal injection: Evaluation of analgesic mechanism

After 40 min acclimatization, control hind paw withdrawal latencies were obtained. Rats (n = 6 per group) were then anaesthetized with sevoflurane (3%) and LASSBio-873, or LASSBio-873 plus methoctramine were administered in 40 μL of vehicle (nanoemulsion) at a dose of 10 μg each. Paw withdrawal latency was measured before and 15, 30, 45 and 60 min after treatment and every 15 min thereafter until rats exhibited complete recovery from analgesia. Antinociceptive effects were determined as a percentage of the maximum possible effect (MPE) according to the following formula:

$$\text{MPE} = [(\text{Post-treatment latency} - \text{Control latency})/ (\text{Cut-off time} - \text{control latency})] \times 100$$

Statistical analysis

Results are expressed as the mean ± SEM. Behavioural data were analysed by two-way analysis of variance (ANOVA) for repeated measures followed by Bonferroni's post hoc correction analysis. Multiple comparisons were made between the control group and the other groups. Circularity was evaluated before ANOVA tests were performed. Differences were considered significant at $P < 0.05$, with a two-sided critical value measure. All tests were performed using GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA, USA).

RESULTS

Dose–response curve

Orally administered LASSBio-873 (8, 20, 40, 75 or 100 mg/kg) produced a dose-dependent analgesic effect in neuropathic animals, as evidenced by increasing withdrawal thresholds (Fig. 1). Withdrawal threshold values in animals treated with 100 mg/kg LASSBio-873 were similar to controls and this dose was used in subsequent experiments.

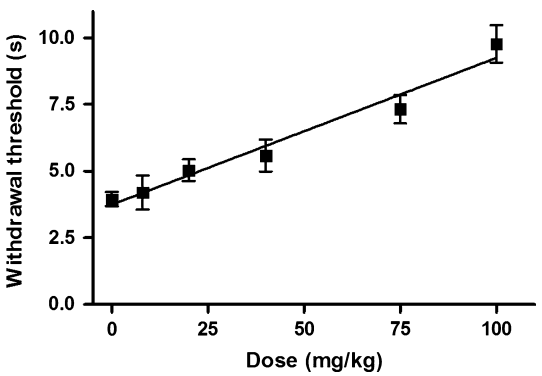

Fig. 1 Dose–response curve for LASSBio-873 in the heat withdrawal test (therapeutic protocol). Seven days after spinal nerve ligation, rats were treated orally by gavage with 0, 8, 20, 40, 75, or 100 mg/kg LASSBio-873 for 7 consecutive days. The paw withdrawal threshold response in the heat hyperalgesia test was evaluated at the end of treatment and latency was plotted using linear regression. The data shown were obtained on the last day of the 7 day treatment period. Data are the mean ± SEM.

Prophylactic protocol

The effects of LASSBio-873 administration on SNL-induced thermal hyperalgesia were evaluated. Sham-operated animals treated with DMSO or LASSBio-873 had no signs of hyperalgesia because withdrawal latency values did not differ between BL and post-surgical Day 7 (Fig. 2). Animals in the SNL group treated with DMSO were hyperalgesic, with a decreased withdrawal latency on post-surgical Day 7 compared with BL (3.9 ± 0.3 *vs* 9.0 ± 0.3 s, respectively; $P < 0.01$). Signs of hyperalgesia were not observed in SNL animals treated with LASSBio-873, with no significant difference between BL and Day 7 after surgery withdrawal latency (10.3 ± 0.7 *vs* 10.9 ± 1.1, respectively; Fig. 2).

The decreased withdrawal latency in the SNL/DMSO group differs significantly from that measured in each of the other groups ($P < 0.01$; Fig. 2).

Effects of LASSBio-873 on SNL-induced mechanical allodynia

As determined by withdrawal threshold measurements, mechanical allodynia did not occur in sham-operated animals treated with either DMSO or LASSBio-873 (Fig. 3). In contrast, the SNL/DMSO group developed allodynia by Day 3 after surgery because withdrawal threshold values decreased from 30.7 ± 1.8 to 21.9 ± 3.4 g and the allodynia persisted until the end of the protocol. LASSBio-873 prevented allodynia in the SNL/LASSBio-873 group because no significant change in withdrawal threshold values occurred from BL to post-surgical Day 7 (34.6 ± 2.3 *vs* 32.2 ± 0.9 g, respectively; Fig. 3).

Therapeutic protocol

Following surgery, sham and SNL animals were observed for 7 days. Animals from the SNL group that developed thermal hyperalgesia and allodynia (as defined by post-surgical withdrawal thresholds significantly lower than BL (control) withdrawal thresholds during the observation period) were treated with vehicle or 100 mg/kg, p.o., LASSBio-873 for 7 days.

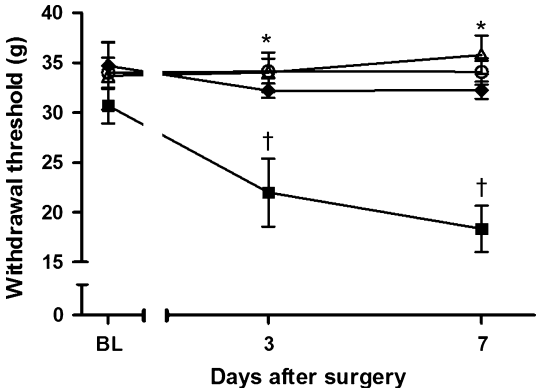

Fig. 3 Evaluation of LASSBio-873 in the mechanical withdrawal test (prophylactic protocol). LASSBio-873 (100 mg/kg, p.o.) was administered daily to sham-operated (○) or spinal nerve-ligated (SNL; ♦) rats for 7 days after surgery. (■), vehicle (dimethylsulphoxide)-treated sham-operated rats; (Δ), vehicle-treated SNL rats. Data are the mean ± SEM. $^{*}P < 0.05$ compared with vehicle-treated SNL rats on the same day; $^{\dagger}P < 0.05$ compared with Day 0 (two-way ANOVA for repeated measures ($k = 9$) followed by Bonferroni's correction post hoc analysis). BL, baseline.

Effects of LASSBio-873 on SNL-induced thermal hyperalgesia

Hyperalgesia, as determined by changes in withdrawal latency, did not occur over the 14 days after surgery in the sham-operated group (Fig. 4). In contrast, all SNL-treated animals developed hyperalgesia by post-surgical Day 7. The SNL/DMSO group had changes in withdrawal latency from 10.2 ± 0.6 to 5.9 ± 0.3 s and this decreased latency persisted for 14 days after surgery (Fig. 4). The SNL/LASSBio-873 group also

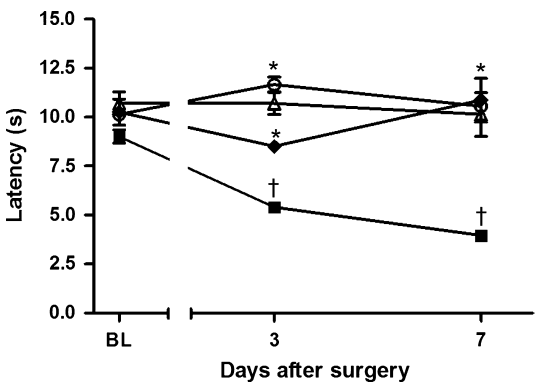

Fig. 2 Evaluation of LASSBio-873 in the heat withdrawal test (prophylactic protocol). LASSBio-873 (100 mg/kg, p.o.) was administered daily to sham-operated (○) or spinal nerve-ligated (SNL; ♦) rats for 7 days after surgery. (■), vehicle (dimethylsulphoxide)-treated sham-operated rats; (Δ), vehicle-treated SNL rats. Data are the mean ± SEM. $^{*}P < 0.05$ compared with vehicle-treated SNL rats on the same day; $^{\dagger}P < 0.05$ compared with Day 0 (two-way ANOVA for repeated measures ($k = 9$) followed by Bonferroni's correction post hoc analysis). BL, baseline.

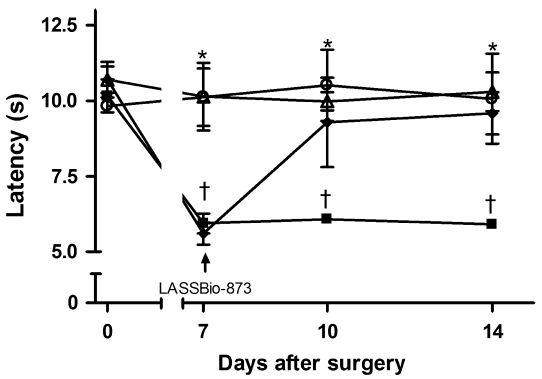

Fig. 4 Evaluation of LASSBio-873 in the heat withdrawal test (therapeutic protocol). LASSBio-873 (100 mg/kg, p.o.) was administered daily to sham-operated (○) or spinal nerve-ligated (SNL; ♦) rats for 7 days after surgery. (■), vehicle (dimethylsulphoxide)-treated sham-operated rats; (Δ), vehicle-treated SNL rats. Data are the mean ± SEM. $^{*}P < 0.05$ compared with vehicle-treated SNL rats on the same day; $^{\dagger}P < 0.05$ compared with Day 0 (two-way ANOVA for repeated measures ($k = 12$) followed by Bonferroni's correction post hoc analysis). BL, baseline.

developed hyperalgesia, with withdrawal latency values changing from 10.7 ± 0.4 to 5.56 ± 0.4 s on post-surgical Day 7. (Fig. 4). LASSBio-873 treatment from Day 7 to Day 14 resulted in a reversal of hyperalgesia with withdrawal latency values on Day 14 (9.6 ± 0.7 s) not significantly different from those obtained during the control period ($P > 0.05$; Fig. 4).

Effects of LASSBio-873 on SNL-induced mechanical allodynia

Withdrawal threshold values did not change in the SHAM/DMSO and SHAM/LASSBio-873 groups from BL throughout the post-surgical period. (Fig. 5). Thus, mechanical allodynia did not occur in these groups. In contrast, allodynia occurred in both SNL groups, as evidenced by decreased withdrawal threshold values by post-surgical Day 7 (Fig. 5). In the SNL/DMSO group, withdrawal threshold declined from 36.6 ± 0.3 to 21.5 ± 1.2 g by Day 7 and remained low through until Day 14. Successful treatment of allodynia by LASSBio-873 was evident by reversal of the withdrawal threshold from 22.6 ± 1.4 g on Day 7 to 33.2 ± 1.0 g on Day 14 (Fig. 5).

All measurements in the contralateral paws were similar across all experimental groups, indicating that treatment with LASSBio-873 according to our protocol (described above) produced no significant motor changes in the animals.

Evaluation of the analgesic mechanism

Intrathecal injection of M_2 receptor antagonist

Intrathecal injection of 10 μg LASSBio-873 produced a maximal antinociceptive effect of 27.1 ± 4.3% MPE 15 min after administration (Fig. 6). This value is similar to the 26.5 ± 5.9% MPE produced by 10 μg morphine, injected as an analgesic reference. Coadministration of LASSBio-873 and methoctramine, an M_2 receptor antagonist, blocked the antinociceptive effects of LASSBio-873 (Fig. 6).

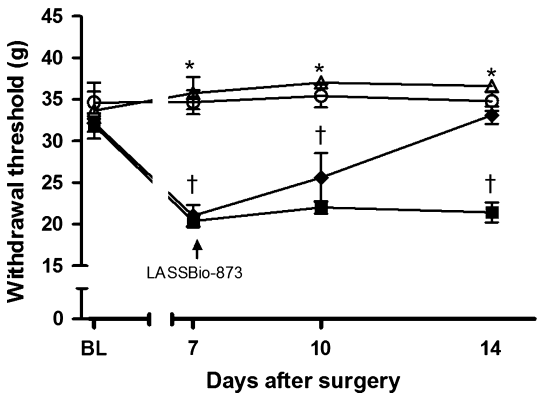

Fig. 5 Evaluation of LASSBio-873 in the mechanical withdrawal test (therapeutic protocol). LASSBio-873 (100 mg/kg, p.o.) was administered daily to sham-operated (○) or spinal nerve-ligated (SNL; ♦) rats for 7 days after surgery. (■), vehicle (dimethylsulphoxide)-treated sham-operated rats; (Δ), vehicle-treated SNL rats. Data are the mean ± SEM. *$P < 0.05$ compared with vehicle-treated SNL rats on the same day; †$P < 0.05$ compared with Day 0 (two-way ANOVA for repeated measures ($k = 12$) followed by Bonferroni's correction post hoc analysis). BL, baseline.

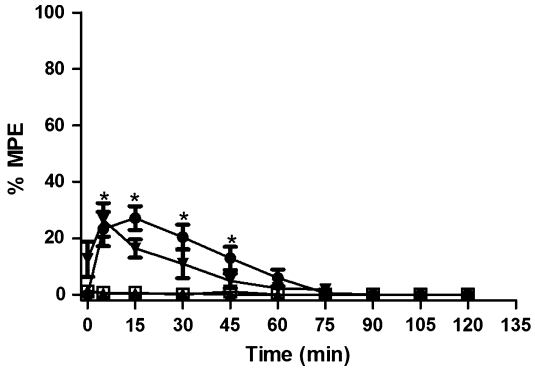

Fig. 6 Time-course of antinociceptive effects showing the percentage maximum possible effect (MPE) of intrathecally administered 10 μg LASSBio-873 (●), 10 μg LASSBio-873 plus 10 μg methoctramine (□), 10 μg morphine (▼) and 40 μL vehicle alone (▲). Control animals received vehicle. Data are then mean ± SEM. *$P < 0.05$ compared with vehicle (one-way ANOVA followed by Dunnett's test for inter-group comparisons).

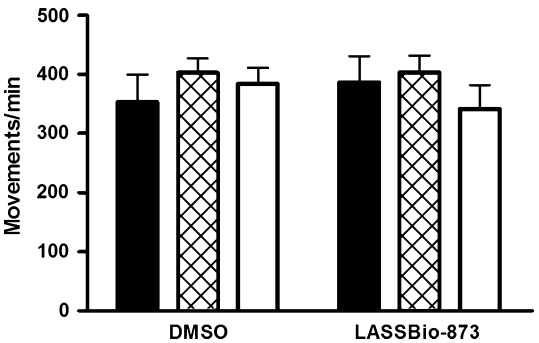

Fig. 7 Locomotor activity after oral administration of LASSBio-873. Motor activity was quantified over a 40 min test session immediately and 24 h after LASSBio-873 administration. (■), 40 min; (▨), 24 h; (□), 7 days. Data are the mean ± SEM of movements per minute. *$P < 0.05$ compared with the control group (dimethylsulphoxide; one-way ANOVA followed by Dunnett's test for inter-group comparisons).

Open field test

The open field test was performed initially before oral administration of the test substance or vehicle. Over a 40 min observation period, the LASSBio-873 (100 mg/kg)- and vehicle-treated groups made similar amounts of movements (35.4 ± 4.6 *vs* 38.7 ± 5.3 movements/min, respectively). Similar results were observed 24 h after LASSBio-873 administration and after 7 days consecutive treatment (40.4 ± 2.9 and 34.1 ± 4.1 movements/min, respectively; Fig. 7).

DISCUSSION

The present study demonstrates that LASSBio-873 is efficacious in preventing and treating neuropathic pain in an SNL animal model. The development of thermal and mechanical hyperalgesia in neuropathic rats was completely abolished by administration of

LASSBio-873. In a therapeutic protocol, LASSBio-873 was also effective because threshold values returned to normal levels, indicating that LASSBio-873 reduces various neuropathic pain symptoms, including thermal hyperalgesia and mechanical allodynia. In addition, our study provides evidence that the analgesic effect of LASSBio-873 seems to be mediated through M_2 receptors, reinforcing the recent findings of Mendes *et al.* in mice.[19] Although a previous study provided evidence for a sedative effect of LASSBio-873,[18] no effect on locomotor behaviour was observed after oral administration of LASSBio-873.

Spinal nerve ligation is a well-characterized model of neuropathic pain and is believed to result in pain sensation similar to that experienced by human patients suffering from neuropathic pain. The SNL model consists of a tight ligation at the L5 spinal nerve distal to the dorsal root ganglion (DRG), which leads to neuropathic pain that is consistent with pain associated with a partial injury of a peripheral nerve. This type of lesion contains both injured and uninjured sensory axons, which induces sympathetically dependent, long-lasting behavioural signs of ongoing pain, including mechanical and heat hyperalgesia, as well as cold allodynia.[20]

Recently, Bartolini *et al.*[24] have shown that different forms of interference in the cholinergic pathway could modulate pain-including direct agonists and substances that promote acetylcholine (ACh) synthesis, enhance ACh release or inhibit acetylcholinesterase activity.

The ability of the M_2 receptor antagonist to block the antihyperalgesic effects of LASSBio-873, as observed herein, supports the hypothesis that the analgesic effect of LASSBio-873 following intrathecal administration is due to activation of M_2 receptors. Similar results were reported by Song *et al.*,[25] who found that the intrathecal administration of oxotremorine, also a muscarinic agonist, produced a significant increase in the tactile withdrawal threshold in rats subjected to partial sciatic nerve lesion by predominantly modulating M_2 muscarinic spinal receptors.

This finding is consistent with established knowledge of cholinergic modulation of peripheral sensory neuronal activity in both neurogenic inflammatory processes and regulation of nociceptive processing directly involving M_2 receptors,[26] the predominant subtype of muscarinic receptor in the spinal cord, as demonstrated in functional studies.[27] In addition, peripheral nerve injury produces various neurobiological events that contribute to chronic pain, including increased expression of M_2 receptors in the DRG.[23]

The spinal cord is the primary site for cholinergic promotion of analgesia. Cholinergic agents block nociception by mimicking the release of ACh from spinal cholinergic nerves.[28] The analgesic effect is produced via activation of both spinal and supraspinal muscarinic cholinoceptors in several species, including humans.[25] Activation of muscarinic cholinoceptors inhibits spinal dorsal horn projection neuronal responses to nociceptive stimuli in rats.[29] The reduction of nociceptive transmission is mediated by the combination of increased inhibitory activity through GABA and glycine release and decreased excitatory activity through inhibition of glutamate release in the spinal dorsal horn. Inhibition of pain signalling likely occurs via M_2 receptors[30] and through activation of lamina II GABAergic interneurons.[31]

Kimura *et al.* have shown that SNL rats, but not normal rats, treated with donezepil (an anticholinesterase agent) exhibited relief of hypersensitivity by increasing extracellular ACh concentrations. This effect was reduced by intrathecal administration of cholinergic and GABAergic antagonists.[32] These findings reinforce our results and the hypothesis that the analgesic effects of LASSBio-873 are mediated through this pathway.

Some authors also demonstrated that activation of supraspinal M_1 receptors is very important for antinociceptive effects of cholinomimetic agents, such as xanomeline[33] and McN-A343,[24] in neuropathic pain. In the present study, the role of M_1 receptor activation in the action of LASSBio-873 was not investigated, but we have demonstrated previously the ability of LASSBio-873 to activate this receptor subtype.[19]

In addition, cholinergic agonists that cross the blood–brain barrier, similar to LASSBio-873, can activate the 'cholinergic anti-inflammatory pathway' and thereby reduce serum levels of proinflammatory cytokines, such as tumour necrosis factor, which then inhibits the antibody response.[29] Inhibition of the inflammatory pathway ultimately leads to a reduction in neuropathic pain symptoms because it is known to have central effects.[18,19]

Numerous studies have demonstrated the importance of muscarinic cholinoceptor agonists in inhibiting neuropathic pain symptoms and cholinomimetics are efficacious in numerous preclinical and clinical pain models, suggesting that they have broad therapeutic potential.[34]

In humans, it is possible that different receptors may be involved in reversing thermal versus mechanical stimuli. However, there appears to be no such dissociation in rodents. Regardless, it would be difficult to conduct direct head-to-head comparisons of potencies between species and differing endpoints given the different measurement methodologies and body areas tested.[35]

Acetylcholine modulates nicotinic receptors and chronic activation of N_1 nicotinic receptors prevents and restores neuropathic injuries. Some nicotinic agonists, such as nicotine and epibatidine, have been shown to have a highly analgesic effect in different models of pain, but most of them have a low therapeutic index.[24]

Development of nicotinic agonists for the management of moderate-to-severe pain has been extensively studied. As yet, no direct nicotinic agonists have been successfully developed and approved for the treatment of neuropathic pain.[36,37]

Other antinociceptive substances, such as clonidine and morphine, also enhance the release of spinal ACh, suggesting a potentially potent role for the spinal muscarinic system in the analgesic effects of these compounds.[30] Substances such as dexmedetomidine, a α_2-adrenoceptor agonist, enhance the withdrawal threshold of SNL rats facilitating KCl-evoked ACh release from lumbar dorsal horn synaptosomes in SNL rats.[34]

The major problem of cholinergic-induced analgesia is the possibility of many side-effects. LASSBio-873 is a novel substance and studies need to be performed to determine whether the side-effects that belong to cholinergic agonists are present in this new chemical class. The findings of the present study also suggest that we can rule out a sedative effect of LASSBio-873 when it is delivered according to our protocols because we found no evidence of a sedative effect after acute oral administration, 24 h after delivery or following 7 consecutive days of treatment. Moreover, over a period of 7 days, rats subjected to SNL exhibited normal behaviour with their contralateral paws. These

findings confirm that the positive effects of LASSBio-873 on neuropathic pain reported in the present study can be attributed to an analgesic effect.

In conclusion, the present study provides evidence of the therapeutic and preventive efficacy of LASSBio-873 via activation of the M_2 receptor pathway in an SNL animal model. This drug belongs to a new group of substances whose efficacy for treating neuropathic pain has not been established. Future elucidation of this compound's pharmacokinetic profile and related pharmaceutical formulations may improve the drug's pharmacokinetic profile and greatly decrease the effective dose. This compound is a novel prototype that may lead to the development of additional novel substances with greater potency for the treatment of neuropathic pain.

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